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Two Purine Biosynthetic Enzymes That Are Required for Cadmium Tolerance in *Schizosaccharomyces pombe* Utilize Cysteine Sulfinate *in Vitro*[1]

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In plants and in certain fungi, exposure to heavy metals induces the synthesis of metal-binding peptides commonly known as phytochelatins. With cadmium, phytochelatins can sequester the metal into a sulfide-containing complex. From genetic analysis of fission yeast mutants, we previously reported that two genes in purine biosynthesis, encoding adenylosuccinate synthetase and succinoaminoimidazole carboxamide ribonucleotide (SAICAR) synthetase, are required for the biogenesis of the phytochelatin–cadmium–sulfide complex *in vivo*. We suggested that a sulfur analog of aspartate, cysteine sulfinate, might be utilized by these enzymes and that the cysteine sulfinate-derived products would then become intermediates or carriers in a sulfur transfer pathway leading to the sulfide found within the metal chelate. In this paper, we report that partially purified adenylosuccinate synthetase and SAICAR synthetase are capable of utilizing cysteine sulfinate *in vitro* to form sulfur analog products. Adenylosuccinate lyase, however, fails to catalyze further conversion of these sulfur derivatives. These observations support the genetic data implicating a link among purine biosynthetic enzymes, sulfur metabolism, and cadmium tolerance. © 1993 Academic Press, Inc.

In response to heavy metal stress, plants and certain fungi synthesize heavy metal binding peptides of the structure $(\gamma\text{-Glu–Cys})_n\text{–Gly}$, where n = 2 to 11. Given names such as cadystins (1), poly (γ-EC)G (2), Cd-binding peptides (3), or γ-glutamyl peptides (4), these peptides have been found throughout the plant kingdom (5) and the term phytochelatins (PCs)[4] (6) has been used most often in recent reviews (7, 8). In plants, PCs have been shown to be synthesized from glutathione (γ-Glu–Cys–Gly) by a constitutively produced enzyme, PC synthase (9). PC synthase activity is induced by heavy metals and termination of PC synthesis is consummated through chelation of the heavy metal cofactors by the newly synthesized peptides (10). Recently, Hayashi *et al.* (11) reported that *Schizosaccharomyces pombe* cell-free extracts can also polymerize $(\gamma\text{-Glu–Cys})_n$ and glutathione to form $(\gamma\text{-Glu–Cys})_{n+1}$ *in vitro*. Addition of a Gly terminal residue can then be catalyzed by glutathione synthetase to form PCs.

In the fission yeast *S. pombe*, the PC–Cd chelates can be resolved into a low molecular weight and a high molecular weight (HMW) complex, with the HMW form containing acid-labile sulfide (12). The incorporation of S^{2-} confers a higher Cd-binding capacity and enhanced stability to the PC–Cd complex (4). Biophysical analysis suggests that the HMW complex consists of a CdS crystallite core with quantum semiconductor characteristics and an outer layer of PC peptides (13). The isolation of fission yeast mutants has firmly established that production of this HMW PC–Cd–S^{2-} complex is essential for a wild-type level of Cd tolerance (14–16). Lacking appropriate mutants, a direct causal relationship has not been

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[4] Abbreviations used: PC, phytochelatin; HMW, high molecular weight; CS, cysteine sulfinate; SAICAR, succinoaminoimidazolecarboxamide ribonucleotide; AIR, aminoimidazole ribonucleotide; AMP-S, adenylosuccinate; CAIR, carboxyaminoimidazole ribonucleotide; AICAR, aminoimidazolecarboxamide ribonucleotide; TLC, thin-layer chromatography; DTT, dithiothreitol; DEAE, diethylaminoethyl; SPA, sulfinylpropanyl adenylate; IU, international unit; PEI, polyethyleneimine.

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established with plants. However, the PC–Cd–S^{2-} complex has been reported to accumulate upon exposure to Cd in *Lycopersicon esculentum* (17) and *Brassica juncea* (18). In addition, sulfide-containing PC–metal complexes are associated with metal-tolerant ecotypes of *Silene vulgaris* (19). These findings suggest that the incorporation of S^{2-} into the metal complex might also have a role in enhanced tolerance in plants, as is the case with *S. pombe.*

We have previously reported on the characterization of two Cd-hypersensitive *S. pombe* mutants that are deficient in the accumulation of the PC–Cd–S^{2-} complex. Analysis of one of the mutants led to the discovery of a vacuolar membrane protein with sequence similarity to a family of membrane pumps (15). Since this putative transporter is required for accumulation of the PC–Cd–S^{2-} complex, formation of this complex appears to require vacuolar compartmentation. Analysis of the other mutant led to the hypothesis that two segments of the purine biosynthesis pathway are involved in heavy metal tolerance (16). Specifically, Cd hypersensitivity and loss of the PC–Cd–S^{2-} complex were observed in mutants deficient in both adenylosuccinate (AMP-S) synthetase (EC 6.3.4.4) and either succinoaminoimidazole carboxamide ribonucleotide (SAICAR) synthetase (EC 6.3.2.6) or aminoimidazole ribonucleotide (AIR) carboxylase (EC 4.1.1.21) (see Fig. 1). A deficiency in AIR carboxylase, SAICAR synthetase, or AMP-S synthetase alone affected neither Cd tolerance nor the accumulation of the PC–Cd–S^{2-} complex.

Biochemical similarity was noted between AMP-S synthetase and SAICAR synthetase as both enzymes catalyze the addition of Asp to the nucleotide intermediate (see Fig. 1). Based on the genetic data, we proposed that either one of these two enzymes is sufficient to catalyze a biochemical step necessary for formation of the PC–Cd–S^{2-} complex. It should be noted that although AIR carboxylase and SAICAR synthetase catalyze reactions of the upstream segment of the pathway, a deficiency of either or both enzymes does not exert an epistatic effect on the downstream segment under normal growth conditions since exogenous adenine, which must be supplemented for growth, regenerates IMP via a salvage pathway. As for a hint of a possible biochemical role for SAICAR synthetase and AMP-S synthetase in the formation of the PC–Cd–S^{2-} complex, Porter *et al.* (20) reported that *in vitro* the *Azotobacter vinelandii* AMP-S synthetase can utilize cysteine sulfinate (CS), a product of cysteine oxidation, in place of Asp in the condensation with IMP. We reasoned that if this analog substrate is utilized by both the *S. pombe* AMP-S synthetase and SAICAR synthetase, then it might be possible that the sulfur analogs of SAICAR and AMP-S are intermediates or carriers in the transfer of S^{2-} to the PC–Cd complex (see Fig. 1).

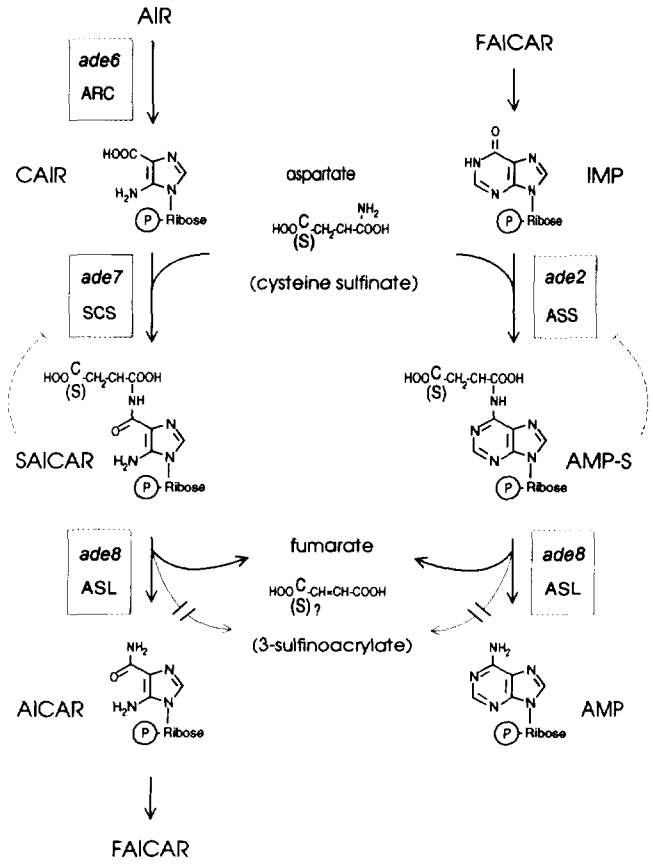

FIG. 1. Purine biosynthetic pathway from AIR to AMP. This linear portion of the purine biosynthetic pathway is arranged to illustrate the two homologous segments involving the reactions catalyzed by AMP-S synthetase, SAICAR synthetase, and AMP-S lyase. Also shown are CS and the hypothetical sulfur analogs. ARC, AIR carboxylase; SCS, SAICAR synthetase; ASL, adenylosuccinate lyase; ASS, adenylosuccinate synthetase.

Since AMP-S lyase (EC 4.3.2.2) catalyzes reactions following both SAICAR synthetase and AMP-S synthetase, a single lesion in the AMP-S lyase-encoding gene should also block both segments of this pathway. Indeed, a mutant deficient in AMP-S lyase was found to be sensitive to Cd. Surprisingly, the mutant retains the capacity to accumulate the PC–Cd–S^{2-} complex, although at a slower rate (16). The reduced rate of HMW complex formation is apparently sufficient to cause Cd sensitivity.

In this study, we report that partially purified AMP-S synthetase from *S. pombe* is capable of utilizing CS as an alternate substrate *in vitro.* Similarly, partially purified SAICAR synthetase can also utilize CS as assayed by thin-layer chromatography. However, we failed to detect further conversion of the S analogs of SAICAR and AMP-S by partially purified AMP-S lyase or crude cell extracts. These observations are compatible with the suggestion that S analogs of SAICAR and AMP-S might be inter-

mediates or carriers in a pathway to form the S^{2-} ion within the PC-Cd-S^{2-} complex. Although AMP-S lyase failed to utilize the S analogs, its absence may result in an accumulation of SAICAR and AMP-S, and hence feedback inhibition of SAICAR synthetase and AMP-S synthetase. A model on the roles of these enzymes in sulfur metabolism and heavy metal tolerance is discussed.

EXPERIMENTAL PROCEDURES

Materials. *S. pombe* strains Sp806 (h *ura4-D18*), B23 (h^+ *ade8.106*), and DS3 (h^- *ura4-D18 ade2::URA3*$^+$) have been described previously (16). Buffers and chemicals were of reagent grade from Sigma Chemical Co., except where indicated. Hadacidin was a gift from Merck Research Chemicals. SAICAR, CAIR, and radiolabeled CS are not commercially available and were synthesized as follows.

Radiolabeled [^{35}S]CS was prepared according to Griffith and Weinstein (21) with the following modifications. One millicurie of L-[^{35}S]cystine (NEN, 300 Ci/mmol) was dried under vacuum and resuspended in 1 ml of formic acid and mixed with 50 mg of nonradiolabeled cystine. After washing overnight with H_2O, the CS was eluted from the Dowex-1 column with a linear gradient of NH_4HCO_3 (300 ml, 0 to 0.1 M). The radioactive peak was collected and dried in a rotary evaporator. The product was monitored by TLC on a silica gel G plates (10 × 10 cm) developed with *t*-butanol:acetic acid:water (75:10:15, v/v/v) and visualized with ninhydrin (0.2%, w/v in ethanol), and was found to have a R_f of 0.2, identical with CS from Sigma. Approximately 30% of the radioactivity was recovered in the final product with a specific activity of 3.6 mCi $mmol^{-1}$.

SAICAR was enzymatically synthesized from AICAR according to Lukens and Flaks (22) with the following exceptions. AICAR (45 mg) was resuspended in 1 ml H_2O and the pH was adjusted to neutral by adding 0.3 ml of 1 N NaOH. Fumarate (257 mg) and AMP-S lyase (~1 IU, enzyme required to transform 1 μmol substrate/min) were added to the AICAR solution. The mixture (brought to 15 ml with H_2O) was incubated at room temperature for 4 h, after which, AMP-S lyase was removed by ultrafiltration (Amicon YM-10). SAICAR was eluted from the Dowex-1 column with a linear HBr gradient (400 ml, 0 to 0.1 N) and detected by its absorbance at 280 nm and by the Bratton-Marshall reaction (see below). Fractions containing SAICAR were pooled and concentrated to ~7 ml in a rotary evaporator and the pH adjusted to 6.5. SAICAR was precipitated with 5 vol of ethanol at −20°C for 3 h, collected by centrifugation, and dried under vacuum. Approximately 24 mg of SAICAR was obtained. Identity was verified by the enzymatic conversion to AICAR via AMP-S lyase (see AMP-S lyase assay) and by TLC (polyethyleneimine cellulose, developed with 1.0 M LiCl) in which an R_f of 0.23 was observed.

CAIR was prepared enzymatically from SAICAR. Enzyme (SAICAR synthetase in the crude extract from the AMP-S lyase-deficient strain B23) was mixed at room temperature in 1 ml of substrate solution (0.5 mM ADP, 10 mM $MgCl_2$, 0.17 mM SAICAR in 20 mM phosphate buffer, pH 7.0). To produce sufficient amounts of CAIR needed for further SAICAR synthetase assays, the reaction was allowed to proceed to completion overnight at 37°C, and CAIR was assayed by the Bratton-Marshall reaction. SAICAR synthetase was removed from the CAIR solution by ultrafiltration.

Detection of purine intermediates. The Bratton-Marshall color reaction (23) was used to monitor purine intermediates using modified incubation temperatures as follows. To assay AICAR and CAIR, 0.1 ml of 4 N HCl was added to 0.1 ml of the reaction mixture and incubated at 37°C for 3 min, followed by the addition of 0.1 ml of sodium nitrite (0.1%, w/v, in 0.01 N HCl) and incubated at 37°C for another 3 min. The reaction was terminated with the addition of 0.1 ml ammonium sulfamate (0.5%, w/v). After 2 min at room temperature, 0.1 ml of naphthylethylenediamine (0.1%, w/v in water) was added to the reaction mixture and incubated for 5 min at room temperature to develop the red product detected at 500 nm. For visualization of SAICAR, the Bratton-Marshall reactions were incubated at 0 to 4°C (detects SAICAR and CAIR) and compared to the reactions incubated at 37°C (detects CAIR only). Beside SAICAR synthetase, AMP-S lyase was able to react with SAICAR to produce AICAR which could also be detected by the Bratton-Marshall reaction. This was avoided by using enzyme prepared from an AMP-S lyase-deficient strain. AMP was measured as described by Park *et al.* (24) where AMP is consumed in a coupled reaction resulting in NADH oxidation and an increase in absorbance at 340 nm. Sample (0.5 ml) was added to an equal volume of a mixture consisting of 30 mM Tris-Cl, pH 8.0, 0.5 mM $MgCl_2$, 50 mM KCl, 0.7 mM ATP, 0.4 mM phosphoenolpyruvate, 0.2 mM NADH, 20 units pyruvate kinase, 10 units of myokinase, and 25 units of lactate dehydrogenase and incubated at room temperature for 1 h.

Purification of AMP-S synthetase, AMP-S lyase, and SAICAR synthetase. The procedure described by Woodward (25) was modified as follows. For AMP-S synthetase, *S. pombe* AMP-S lyase-deficient strain B23 was grown in rich medium (YG) to near saturation (15). After centrifugation, the cell pellet (~40 g) was resuspended in 2 vol (~80 ml) of 10× PB (0.2 M sodium phosphate, pH 7.0, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) and transferred to the blending chamber of the Bead Beater (Biospec Products). All operations were performed on ice. An equal volume of 0.5-mm glass beads was added and the cell mixture subjected to 10 1-min cycles of blending interrupted by 1 min cooling periods. Cell lysis was monitored under the microscope. The extract was collected by filtration through Miracloth, and the beads were washed with an additional 50 to 100 ml of 10× PB. Protamine sulfate was then added slowly to the combined extract to a final concentration of 2 mg ml^{-1} and allowed to stir for an additional 15 to 30 min on ice. The supernatant was collected after centrifugation (15,000*g*, 15 min, 4°C) and fractionated by the addition of powdered ammonium sulfate (40 to 70% saturation, added over 5 min and stirred an additional 30 min at 0°C). The precipitate collected by centrifugation was dissolved in a minimum amount of Tris-Cl buffer (10 mM, pH 7.5, 0.1 mM EDTA, 0.1 mM DTT) and desalted by gel filtration through Sephadex G25m (Pharmacia) equilibrated in Tris-Cl buffer. The crude enzyme preparation was then fractionated by anion-exchange (DEAE Sephacel, Pharmacia) column chromatography (2.6 × 18.5 cm, equilibrated in the same Tris-Cl buffer). The column was washed with Tris-Cl buffer until the baseline absorbance at 280 nm was obtained, and then the protein was eluted with a linear salt gradient (500 ml, 0 to 0.5 M NaCl in Tris-Cl buffer). The fractions containing AMP-S synthetase activity were pooled, subjected to buffer exchange into 1× PB in a large G25m column, concentrated by ultrafiltration (YM-10, Amicon), and loaded onto a hydroxylapatite (Bio-Gel HPT, Bio-Rad) column (0.9 × 7 cm, equilibrated in 1× PB). After washing with 20 ml of 1× PB, the enzyme was eluted by a linear gradient of sodium phosphate (200 ml, 0 to 0.2 M in 1× PB). The enzyme was stored in 50% (v/v) glycerol at −20°C. No noticeable loss of activity was observed over a period of 6 months. The Bio-Rad dye-binding assay with bovine serum albumin as a standard was used to determine protein concentration.

The same purification procedure described above for AMP-S synthetase was used to purify AMP-S lyase from the AMP-S synthetase-deficient strain DS3. As with AMP-S synthetase, AMP-S lyase was stable in 50% (v/v) glycerol at −20°C for at least 6 months.

For SAICAR synthetase, the enzyme was prepared from the AMP-S lyase-deficient strain B23 with the above procedure except for the following modifications. The ammonium sulfate precipitate collected by centrifugation was dissolved in a minimum amount of 1× PB and dialyzed (12,000 to 14,000 *M*, cutoff, SpectraPor) in the same buffer overnight at 4°C with two changes of the dialysate. After a brief centrifugation to remove the precipitate formed during dialysis, the crude enzyme preparation was fractionated by anion-exchange (DEAE Sephacel, Pharmacia) column chromatography (2.6 × 18.5 cm, equilibrated in 1× PB). The column was washed with 1× PB (~100 ml) and the protein was eluted with a linear salt gradient (500 ml, 0 to 0.5 M NaCl in 1×

TABLE I

Purification of AMP-S Synthetase from *S. pombe* Strain B23 (AMP-S Lyase Deficient)

Fraction	Activity (nmol · min^{-1} mg^{-1})	Total units (μmol · min^{-1})	Fold purification	Recovery (%)
Crude extract	7.8	11.0	1.0	100
Protamine sulfate	7.3	7.1	0.9	65
Ammonium sulfate	9.7	4.6	1.2	41
Anion exchange	38	7.0	4.8	64
Hydroxylapatite	340	7.1	44	65

PB). The fractions containing SAICAR synthetase activity were collected, concentrated by ultrafiltration (YM-10, Amicon), and loaded onto the hydroxylapatite column. No noticeable loss of activity was observed over a period of 6 months when stored in 50% (v/v) glycerol at −20°C.

Enzyme assays. AMP-S synthetase was assayed as described (26). Enzyme was mixed at room temperature in 1 ml of substrate solution containing 0.4 mM IMP, 0.1 mM GTP, 8.0 mM magnesium acetate, 50 mM Hepes, pH 7.0, and 8.0 mM Asp or CS unless otherwise specified in the text. Activity, the increase in absorbance at 280 nm, was monitored in a dual beam spectrophotometer (Shimadzu UV 160), compared to a reference reaction without Asp or CS. Specific activity was calculated using the molar extinction coefficient of 11,700 M^{-1} cm^{-1} (26), expressed as IU/mg protein. All assays with radiolabeled CS in place of Asp (including those with AMP-S lyase and SAICAR synthetase, see below) were scaled down to 0.1 ml and contained 8.8 mM [^{35}S]CS (3.2 μCi). Radiolabeled substrates and products were analyzed by TLC according to methods used to separate monophosphorylated purines (27). Generally, 20 to 40 μl of the reaction mixture was spotted on the polyethyleneimine cellulose plate (PEI, Sigma T6765, with fluorescent indicator, 20 × 20 cm). Plates were developed in a chromatographic chamber with 1 M LiCl for 3 to 4 h, air dried, covered with plastic wrap, and subjected to autoradiography at room temperature for 48 h.

For AMP-S lyase, the enzyme was mixed at room temperature with 1 ml of substrate solution (70 μM AMP-S in 50 mM Tris-Cl buffer, pH 8.0) and the decrease in A_{280}, compared to a reference reaction without AMP-S, was recorded. Specific activity was calculated using the molar extinction coefficient of 10,700 M^{-1} cm^{-1} (25).

For partial purification, SAICAR synthetase activity was measured in the reverse direction as described above for CAIR preparation. The reactions were allowed to proceed for 1 h at 37°C and CAIR formation was detected by the Bratton-Marshall assay. Activity was estimated using the molar extinction coefficient of 15,000 M^{-1} cm^{-1} (28). For the forward reaction, 8 mM Asp and 0.7 mM ATP were added to 1 ml of the CAIR solution (prepared as described), the reaction was allowed to proceed at 37°C, and the products were monitored using the Bratton-Marshall assays. When [^{35}S]CS (3.2 μCi) was used in place of Asp, the products of the forward reaction were analyzed by TLC as described above. The forward reaction with [^{35}S]CS was performed in a volume of 0.1 ml of the CAIR solution to which was added [^{35}S]CS and ATP to a final concentration of 8.8 and 0.7 mM, respectively.

RESULTS

Partial purification of the enzymes. AMP-S synthetase, AMP-S lyase, and SAICAR synthetase were partially purified by ammonium sulfate fractionation, DEAE Sephacel ion-exchange, and hydroxylapatite chromatography. In experiments with the wild type *S. pombe* strain Sp806, AMP-S synthetase and AMP-S lyase could be separated by DEAE ion-exchange on an analytical scale (data not shown), with AMP-S synthetase eluting at lower NaCl concentrations than AMP-S lyase. However, the separation was not sufficient in preparative work to ensure a complete lack of cross contamination of the two enzymatic activities. Starting with ~40 g (wet weight) of cells, 7 IU of AMP-S synthetase was obtained from the AMP-S lyase-deficient mutant B23 and 12 IU of AMP-S lyase was obtained from the AMP-S synthetase-deficient mutant DS3. Tables I and II present the results of AMP-S synthetase and AMP-S lyase purification, respectively. In a separate experiment with the AMP-S lyase-deficient mutant B23, ~1.4 IU of SAICAR synthetase was obtained. The activity of SAICAR synthetase eluted near the middle of the NaCl gradient in the DEAE ion-exchange and it flowed directly through the hydroxylapatite column. Since the assay conditions for SAICAR synthe-

TABLE II

Purification of AMP-S Lyase from *S. pombe* Strain DS3 (AMP-S Synthetase Deficient)

Fraction	Activity (nmol · min^{-1} mg^{-1})	Total units (μmol · min^{-1})	Fold purification	Recovery (%)
Crude extract	28	34	1.0	100
Protamine sulfate	25	30	0.9	87
Ammonium sulfate	45	18	1.6	53
Anion exchange	420	16	15	46
Hydroxylapatite	3000	12	106	35

tase with Asp required long incubation times, linearity could not be guaranteed and activity can only be estimated, therefore a purification table for SAICAR synthetase is not presented. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the various enzyme preparations revealed multiple bands, indicating that they were not yet homogeneous. However, the *in vitro* catalytic specificity of each synthetase remained distinct for its respective *in vivo* substrates (data not shown).

Biochemical characterization AMP-S synthetase. Binding affinities and maximum velocities of AMP-S synthetase were calculated for both Asp and CS as substrates. Figure 2A is the Lineweaver–Burk plot (velocity versus substrate concentrations in inset) of AMP-S synthetase using Asp as a substrate. The K_m for Asp calculated from this plot is 1.12 mM. This agrees with the previously reported value of 1.5 mM as the K_m for Asp of AMP-S synthetase from *S. pombe* (29). The apparent maximum velocity calculated of this enzyme preparation is 1.87 mmol min^{-1} mg^{-1}. Figure 2B presents the corresponding characterization using CS as the substrate. The resulting K_m and apparent maximum velocity calculated from this plot are 10.1 mM and 250 nmol min^{-1} mg^{-1}, respectively. Due to difficulties for quantitation of CAIR from the forward or reverse reactions, and the interference of CS in the Bratton–Marshall reaction, SAICAR synthetase was not further characterized for its substrate-binding affinity.

In vitro reactions with AMP-S synthetase and AMP-S lyase. The AMP-S synthetase AMP-S lyase coupled reaction using Asp as the substrate is shown in Fig. 3A. The production and consumption of the intermediate, AMP-S, was calculated from the change in absorbance at 280 nm. The AMP-S synthesized from IMP and Asp by the AMP-S synthetase reaction in the first 15 min was quantitatively converted to AMP by the addition of an equal number of units of AMP-S lyase to the reaction. When Asp was replaced with CS in the AMP-S synthetase reaction (Fig. 3B), the A_{280} also increased suggesting that the S analog sulfinylpropanyl adenylate (SPA) was formed. After 15 min, SPA accumulated to 28% the level of AMP-S, as calculated with the molar extinction coefficient for AMP-S. It should be noted that in these reactions, excess AMP-S synthetase activity was used to maximize product formation, thus the reactions were not linear. At lower concentrations of enzyme, the reaction of AMP-S synthetase with either Asp or CS was linear with both time and amount of enzyme (data not shown). As in the case of Asp, this CS-dependent increase in A_{280} was not observed when IMP, GTP, or Mg^{2+} was omitted from the reaction (not shown). In contrast to the coupled reaction with Asp as a substrate, addition of AMP-S lyase to this reaction failed to reduce the A_{280} and instead a

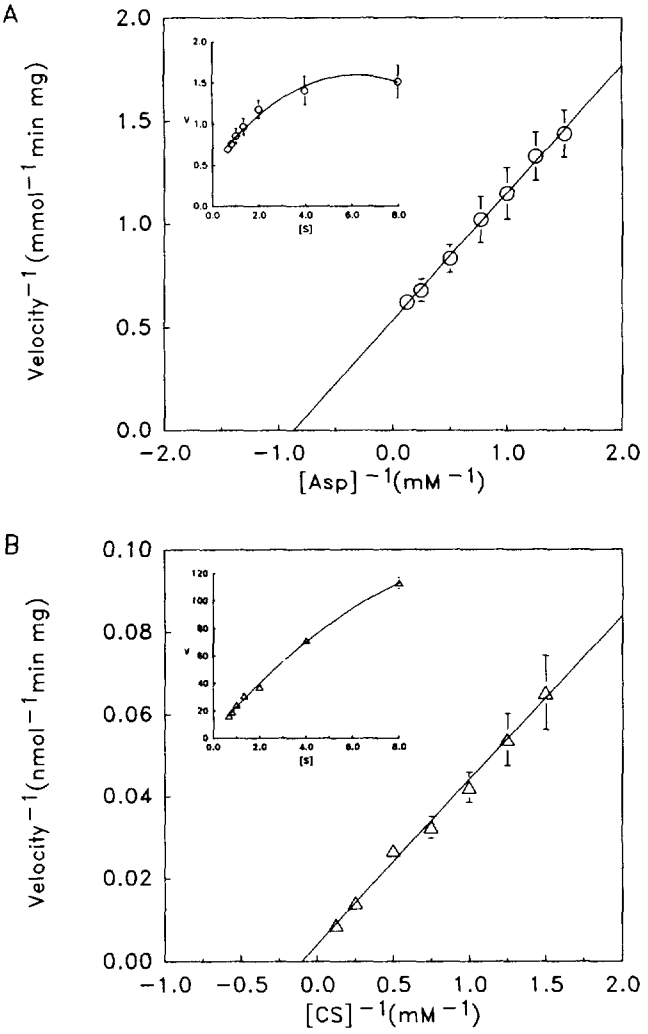

FIG. 2. Initial velocity patterns (inset) and double reciprocal plots for AMP-S synthetase. (A) Double reciprocal plot for the AMP-S synthetase varying Asp concentration (○). (B) Double reciprocal plot for the AMP-S synthetase varying CS concentration (△). All points represent the average of three determinations, error bars represent 1 SD.

steady increase was observed (Fig. 3B). To examine the possibility that a lack of observable reaction by AMP-S lyase (and a lack of feedback inhibition of AMP-S synthetase) might be due to a lower substrate concentration, a longer AMP-S synthetase reaction with CS and IMP was performed (Fig. 3C). After 3 h incubation, the amount of SPA produced was comparable to the ~60 nmol of AMP-S observed in Fig. 3A and begins to exhibit hyperbolic kinetics. In addition to the higher amount of SPA available as substrate, AMP-S synthetase activity was removed from the reaction mixture by ultrafiltration prior to the addition of AMP-S lyase. This alteration failed to produce a reduction in the A_{280}.

Since a formal possibility existed that an isoform of AMP-S lyase might be present in Cd-stressed cells that

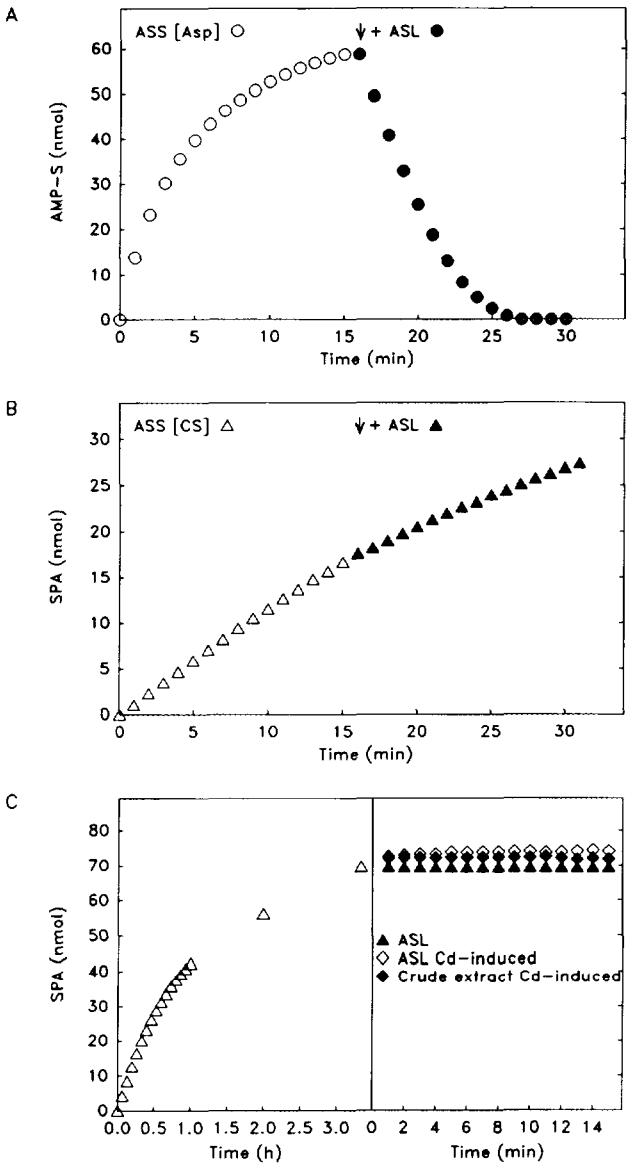

FIG. 3. The *in vitro* reactions of AMP-S synthetase (ASS) and AMP-S lyase (ASL) monitored spectrophotometrically. (A) The AMP-S synthetase AMP-S lyase reaction using Asp as the substrate (○, ●). (B) The alternative AMP-S synthetase AMP-S lyase reaction using CS as the substrate (△, ▲). (C) A longer reaction of AMP-S synthetase with CS (△), with subsequent removal of AMP-S synthetase by ultrafiltration and followed by the addition of partially purified AMP-S lyase from a nonmetal-induced culture (▲), partially purified AMP-S lyase from a 50 μM Cd-induced culture (◇), or a crude protein extract from a 50 μM Cd-induced culture (◆). Nonlinear reactions with AMP-S synthetase and AMP-S lyase (17 mIU of each) were used to maximize product formation.

is not found in nonstressed cells, crude extract and AMP-S lyase were prepared from the AMP-S synthetase-deficient strain grown in the presence of 50 μM Cd. However, the addition of neither the partially purified AMP-S lyase (ammonium sulfate fraction) nor crude extract from Cd-induced cultures caused a change in the A_{280} (Fig. 3C), although, as in Fig. 3A, both sources of the enzyme caused the expected drop in A_{280} when AMP-S was used as a substrate (not shown). This suggests that at least *in vitro* the S analog of AMP-S is not metabolized by AMP-S lyase. The formal possibility that SPA is not recognized by our AMP-S lyase preparation due to the loss of cofactor(s) during the enzyme purification steps is still possible, although that does not seem probable given the lack of a reaction observed with crude extracts.

Although the spectrophotometric assay for AMP-S lyase was informative, the conclusion that SPA is not further metabolized rests on the assumption that the reaction products derived from SPA, if any, would exhibit a change in absorbance at 280 nm. Since this might not be valid, two alternative assays were conducted. First, we measured the production of AMP, an expected product of the AMP-S synthetase AMP-S lyase coupled reaction. Whereas AMP was indeed produced when Asp was provided as the substrate, no AMP was detected when CS was used as a substrate (not shown). In the second assay, [^{35}S]CS was used as the substrate for the AMP-S synthetase AMP-S lyase reactions and the radiolabeled product was monitored after fractionation by TLC. The autoradiogram in Fig. 4A shows that a new spot with an R_f of 0.24 (presumably SPA) was produced by the AMP-S synthetase reaction (lane 2). This spot has an R_f value distinct from AMP-S (R_f = 0.3) and is not observed with the use of heat-denatured AMP-S synthetase (lane 1). Subsequent addition of active (lane 4) or heat-inactivated AMP-S lyase (lane 3) to the reaction failed to reduce the signal at the position of the putative S analog or to create a new spot predicted by the release of 3-sulfinoacrylate. The same holds true with the addition of crude extract to the reaction (not shown). The production of the putative SPA spot was inhibited by adding Asp to the AMP-S synthetase reaction, which should act as a competitor to [^{35}S]CS (Fig. 4B, lane 2). Addition of hadacidin, a specific inhibitor of AMP-S synthetase (30), also abolished the formation of this new product (Fig. 4B, lane 4), suggesting that the new product is formed specifically by AMP-S synthetase and not by a contaminating enzyme present in the reaction. Omitting IMP (lane 3) or Mg^{2+} (not shown) had the same effect. These results are consistent with the interpretation that the new spot is indeed a product specifically catalyzed by AMP-S synthetase utilizing [^{35}S]CS as the substrate and is most likely SPA. As in the enzyme assay based on A_{280} or on the measurement of AMP production, no further enzymatic conversion on the putative SPA spot was observed by the subsequent addition of AMP-S lyase or crude extract.

In vitro reactions with SAICAR synthetase and AMP-S lyase. Because of the lability of most metabolites in

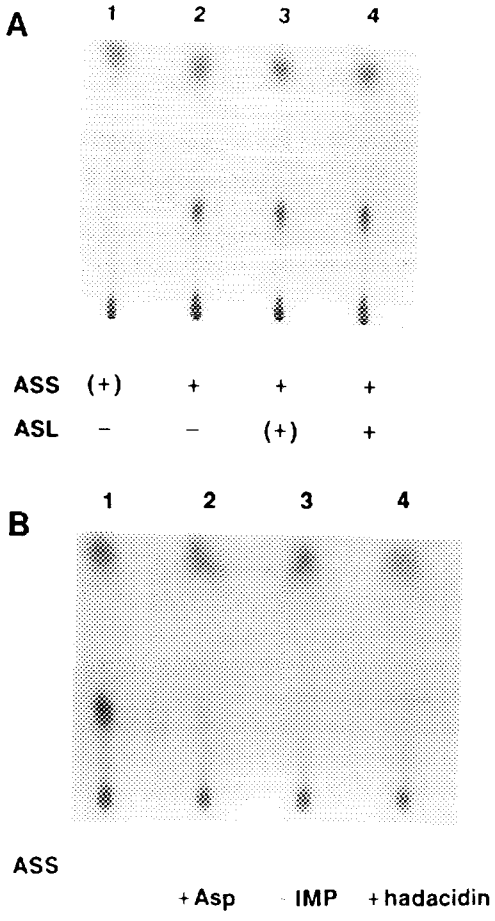

FIG. 4. TLC and autoradiography of the AMP-S synthetase AMP-S lyase reaction using radioactive cysteine sulfinate as the substrate. In all reactions, 7×10^6 cpm of [^{35}S]CS were added to the 0.1 ml AMP-S synthetase (0.3 mIU) reaction, with subsequent additions as described in the legends to the Figures. (A) The autoradiogram of the AMP-S synthetase reaction showing a new spot appearing (lane 2). Boiled enzyme (indicated in parentheses) prevented production of this spot (lane 1). However, subsequent overnight incubation with 0.4 mIU of AMP-S lyase, boiled or not, failed to change the mobility of the putative sulfur analog (lanes 3 and 4, respectively). (B) To verify that the new spot is the product of CS specifically catalyzed by AMP-S synthetase, the reaction mixture was modified as follows: the addition of Asp (16 mM) as a competitive substrate (lane 2); the addition of hadacidin (14.3 mM), a specific inhibitor of AMP-S synthetase activity (lane 4), and exclusion of IMP (lane 3) from a 4-h reaction.

the IMP biosynthetic pathway, the substrate and product of the SAICAR synthetase reaction, CAIR and SAICAR, are not available from commercial sources. Therefore SAICAR was synthesized by AMP-S lyase in the reverse reaction from AICAR and fumarate, and CAIR was synthesized by SAICAR synthetase in the reverse direction from SAICAR and ADP. Partially purified SAICAR synthetase was used to determine the utilization of CS as a substrate. The purine intermediates CAIR, SAICAR, and AICAR could be detected by the Bratton–Marshall reaction, and they were distinguishable by altering the reaction temperature. Unfortunately, the color reaction was strongly inhibited by CS (not shown), and we were not able to monitor the SAICAR synthetase/AMP-S lyase reaction spectrophotometrically when CS (in place of Asp) was the substrate. Therefore, we examined the reaction products by autoradiography following TLC.

In the SAICAR synthetase reaction, [^{35}S]CS was added to CAIR and the reaction was then monitored over a period up to 6 h (Fig. 5A). A smeared spot (R_f = 0.16) ap-

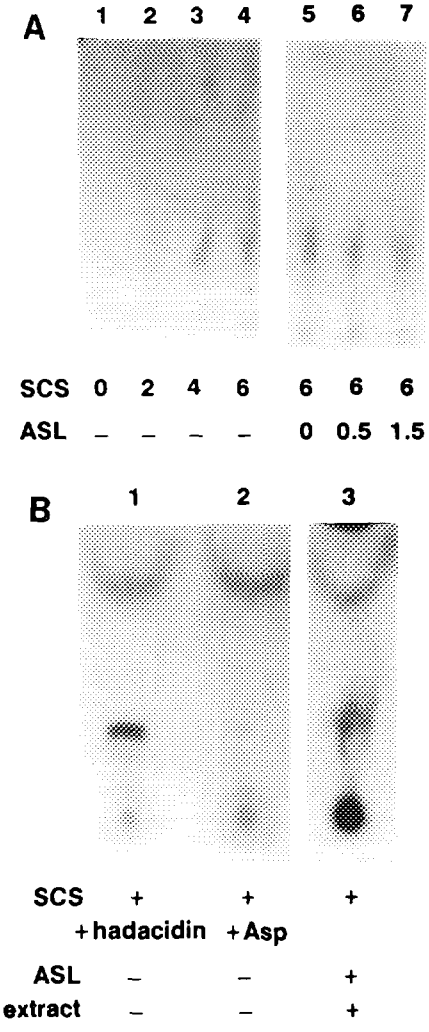

FIG. 5. TLC and autoradiography of the SAICAR synthetase AMP-S lyase reaction using radioactive cysteine sulfinate as the substrate. (A) The SAICAR synthetase reaction (~0.5 mIU enzyme for 2, 4, or 6 h) generated a novel spot that represents the putative sulfur analog of SAICAR (lanes 2–4). However, subsequent incubation after the addition of AMP-S lyase for 0.5 or 1.5 h failed to change the mobility of the putative analog (lanes 6 and 7). (B) The SAICAR synthetase reaction was not inhibited by 14.3 mM hadacidin (lane 1), but product formation was subject to competition by the addition of 16 mM Asp to the reaction (lane 2). The subsequent addition of AMP-S lyase and crude extract failed to cause any change in the position of the ^{35}S-product (lane 3).

peared gradually which we presume to be the S analog of SAICAR (R_f = 0.23). No change was observed upon further addition of AMP-S lyase for up to 90 min (Fig. 5A, lanes 6 and 7) or longer (not shown). It is unclear what caused the smearing of the spots. As with the AMP-S synthetase reaction, Asp also inhibited the incorporation of radioactive CS into the new spot, as expected for a competitive substrate (Fig. 5B, lane 2). Also observed was the lack of inhibition shown by hadacidin, which should only affect the activity of AMP-S synthetase and not SAICAR synthetase (lane 1). This also confirms that the hadacidin inhibition shown in Fig. 4B, lane 4 is not due to a factor that inhibits the activities of enzymes in general. Finally, to examine whether the lack of AMP-S lyase activity on this putative S analog might be due to the lack of a cofactor, crude extract alone (not shown) or together with AMP-S lyase (lane 3) was added subsequent to the SAICAR synthetase reaction. In both cases, no effect on the signal for the putative SAICAR analog was observed. These observations suggest that SAICAR synthetase can indeed incorporate CS in place of Asp, but as with the S-substituted product from an AMP-S synthetase-catalyzed reaction, the S-substituted product from the SAICAR synthetase reaction also failed to be metabolized by AMP-S lyase.

DISCUSSION

We have recently described the isolation of a Cd-sensitive mutant, LK69, that fails to accumulate wild-type levels of the PC–Cd–S^{2-} complex (16). Derived from a genetically marked strain lacking AIR carboxylase activity, LK69 was found to also harbor a lesion in the AMP-S synthetase-encoding gene. Since adenine was provided as a growth supplement for strains derived from the progenitor, this additional defect in the purine biosynthetic pathway was not discovered until the molecular identification of the functional cDNA. Because neither a single deficiency of AIR carboxylase nor AMP-S synthetase affected Cd tolerance (and formation of the PC–Cd–S^{2-} complex), we deduced that each segment of the pathway must be capable of complementing each other, as for instance in the catalysis of a common or similar reaction. Indeed, both SAICAR synthetase and AMP-S synthetase catalyze biochemically analogous reactions. Further analysis of a mutant blocked in the activities of SAICAR synthetase and AMP-S synthetase, which has the same Cd-sensitive phenotype, lends support to this argument. Thus, from this serendipitous isolation of a mutant with an additional defect in the purine biosynthesis pathway, we found a relationship between mutant purine biosynthetic genes and Cd sensitivity. The present paper addresses whether this causal effect could be due to a participation of these enzymes in sulfur metabolism.

A possible role in sulfur metabolism. Fission yeast exhibits up to 40-fold higher levels of acid-labile sulfide during Cd stress as compared to control cultures, presumably due to the formation of the stable peptide–Cd–S^{2-} complex. Lower levels of cellular S^{2-} were indeed found with the mutants that lack SAICAR synthetase and AMP-S synthetase or AIR carboxylase and AMP-S synthetase (16). Although this correlation did not necessarily indicate a causal relationship, it nonetheless prompted us to consider whether these two segments of the purine biosynthesis pathway are involved in Cd-induced S^{2} production. We proposed that SAICAR synthetase and AMP-S synthetase might utilize CS in place of Asp in their respective reactions. If this were possible, then it could be envisioned that the S analogs of SAICAR and AMP-S might be intermediates or carriers in a sulfur transfer pathway leading to the S^{2-} found in the stable metal chelate. In this work, we have shown that fission yeast AMP-S synthetase is capable of this reaction *in vitro*. The partially purified enzyme from *S. pombe* can indeed utilize CS, similar to the *A. vinelandii* enzyme reported by Porter *et al.* (20). Their analysis was based not on the formation of a S derivative product, but on measurements of GTP hydrolysis. In addition to our spectrophotometric assays, which indicated product formation, we also detected a S derivative product on TLC. Moreover, product formation requires IMP, CS, GTP, Mg^{2+}, and AMP-S synthetase. Finally, its formation can be abolished by the AMP-S synthetase inhibitor hadacidin. These observations support the contention that the product is SPA, the S analog of AMP-S.

In the case of SAICAR synthetase, there has not been a previous report of CS utilization of which we are aware. The lack of commercially available substrates for this reaction, combined with the interference by CS of the Bratton–Marshall color reactions, made it more difficult to purify and assay this enzyme. With the use of radiolabeled CS, we have also been able to show the formation of a S derivative product that is likely to be the S analog of SAICAR.

Physiological relevance. As determined *in vitro,* the substrate affinity of AMP-S synthetase for Asp versus CS differs by almost an order of magnitude, and this questions whether AMP-S synthetase (and SAICAR synthetase) could be important in CS utilization *in vivo.* To address this issue, we first consider whether the S analogs of SAICAR and AMP-S are carriers or donors. If they are carriers, then the sulfide in the PC–Cd–S^{2} complex is derived from a S atom of another source, and the carriers need not be synthesized in large numbers. If the S analogs are donors, however, we would have to consider how they may be produced in high amounts. One possibility is that the *in vitro* determined K_m values may not be meaningful during Cd stress. Since cell growth is arrested by Cd, *de novo* synthesis of purines may not be critical; hence, Asp utilization by AMP-S synthetase and SAICAR synthetase

may not be the highly competitive reactions as suggested by the *in vitro* K_m values.

An indirect role for AMP-S lyase. If the S analogs of SAICAR and AMP-S are indeed synthesized *in vivo*, then the obvious question is whether AMP-S lyase is involved in conversion of these analogs to 3-sulfinoacrylate, the S analog of fumarate. Should this be the case, then we would predict that a lesion in the AMP-S lyase-encoding gene would cause sensitivity to Cd along with diminished production of the PC–Cd–S^{2-} complex. When we examined such a mutant (16), it was indeed Cd-sensitive. Inconsistent with this prediction, however, was the observation that the mutant retains the capacity to form the PC–Cd–S^{2-} complex, although at a slower rate. The reduced rate of HMW complex accumulation is apparently sufficient to cause Cd sensitivity. Assuming that the *in vivo* incorporation of CS into S analogs of AMP-S and SAICAR is involved in Cd tolerance, two explanations could account for this apparent anomaly with the AMP-S lyase-deficient strain.

In one interpretation, AMP-S lyase activity is needed for cleavage of the S analogs of AMP-S and SAICAR to yield the S analog of fumarate. This would implicate 3-sulfinoacrylate as a metabolic precursor or carrier of the acid labile S^{2-} found in the metal complex, and the AMP-S lyase-deficient mutant's capacity to gradually accumulate the HMW complex might be attributed to a leaky mutant allele. However, less than 0.1% of the wild-type level of AMP-S lyase activity in a crude cell extract from this mutant was observed (data not shown). Although it is difficult to determine what level of enzyme activity could be considered "leaky," it nonetheless does not lend support for this interpretation.

In previous work AMP-S lyase was unable to catalyze a further reaction with the nitro analog of AMP-S formed by AMP-S synthetase from *A. vinelandii* (20). Similarly, our data indicate that AMP-S lyase cannot utilize these putative S analogs as substrates, suggesting that it is the formation of the S derivatives and not their conversion by AMP-S lyase that is required for the ultimate production of sulfide. Since it is known that AMP-S feedback inhibits the activity of AMP-S synthetase from several bacterial sources (31), a second interpretation is that a build-up of AMP-S resulting from a lack of AMP-S lyase activity might be inhibiting AMP-S synthetase activity and preventing effective addition of CS to IMP. In this latter interpretation, an assumption is made that there would also be feedback inhibition of SAICAR synthetase by a build-up of SAICAR. The lack of AMP-S lyase activity on these S derivatives is at least compatible with this latter interpretation. Assuming that feedback inhibition is less effective than genetic blockage of enzyme synthesis, this might account for the apparent "leakiness" in the slow accumulation of the PC–Cd–S^{2-} complex seen in the AMP-S lyase-deficient mutant.

A working model. The role for each of these three enzymes in the accumulation of the PC–Cd–S^{2-} complex is illustrated in Fig. 1. CS, derived from Cys by cysteine dioxygenase, is incorporated into either sulfinylpropanylaminoimidazolecarboxamide ribonucleotide or SPA by SAICAR synthetase or AMP-S synthetase, respectively. The loss of SAICAR synthetase activity alone, blocking formation of IMP from CAIR, does not affect the availability of IMP when cells are supplemented with IMP-regenerating adenine. AMP-S lyase does not utilize the S analogs, but its presence is nonetheless necessary. In the absence of AMP-S lyase, a build-up of SAICAR and AMP-S feedback inhibits their respective synthetases, producing a phenotype that mimics genetic lesions in both the SAICAR synthetase and AMP-S synthetase encoding genes. Presumably, the S analogs are either donors or carriers that lead to formation of the PC–Cd–S^{2-} complex necessary for Cd tolerance. Although the data we have obtained thus far are compatible with the above model, an alternative hypothesis that cannot be excluded is that SAICAR or AMP-S (or AMP-S synthetase or SAICAR synthetase) is required as an essential activator or cofactor of another enzyme(s) involved in sulfate assimilation or sulfide transfer. Validity of the working model awaits the characterization of additional mutants deficient in accumulation of the PC–Cd–S^{2-} complex.

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